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Quantification of plasma and egg 4,4′-dinitrocarbanilide (DNC) residues for the efficient development of a nicarbazin-based contraceptive for pest waterfowl†

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Abstract: Urbanization and associated landscaping has increased the abundance of year-round habitat for waterfowl, resulting in vegetation damage, loss of recreational activities, air transportation mishaps and health hazards. As part of a research program to develop socially acceptable techniques for management of pest bird populations, we are evaluating nicarbazin as a contraceptive in pest and surrogate avian species. As reproductive studies with Canada Geese (*Branta canadensis*) are tedious due to the difficulty of conducting controlled field studies and/or breeding geese in captivity, we evaluated the effects of oral nicarbazin administration on the production and hatchability of chicken eggs. Blood plasma and egg DNC concentrations were correlated to contraceptive efficacy. Subsequent studies are being conducted with geese to determine the diet nicarbazin concentration required to produce the desired blood and plasma DNC concentrations. This approach permits the expeditious evaluation of formulations and dosing regimes by simply monitoring blood DNC concentrations in target species.

Keywords: nicarbazin; 4,4′-dinitrocarbanilide; DNC; geese; contraception

1 INTRODUCTION

Urban and suburban conflicts between man and wildlife are increasing in frequency. This is partially attributable to a decrease in wildlife habitat as growing residential areas engulf former wildlife habitat. In addition, landscaping to form parks and corporate campuses has increased the abundance of year-round habitat for waterfowl. This has contributed to increasing year-round urban resident populations of migratory waterfowl species such as mallards (*Anas platyrhynchos* L) and Canada geese (*Branta canadensis* L). These populations are frequently associated with vegetation damage, loss of recreational activities and health hazards due to the deposition of large quantities of excreta.

In such situations, population reduction by lethal means is unacceptable to a significant proportion of society. Relocation of pest waterfowl is frequently impracticable as relocation sites are generally unavailable. In an effort to develop socially acceptable techniques to manage pest bird populations, the National Wildlife Research Center (NWRC) is evaluating several compounds as potential contraceptives. One such compound is nicarbazin, an equimolar complex of 4,4′-dintrocarbanilide (DNC) and 4,6-dimethyl-2-pyrimidinol (HDP) (Fig 1).

Nicarbazin, a US Food and Drug Administration approved anticoccidial agent, has been used in broiler

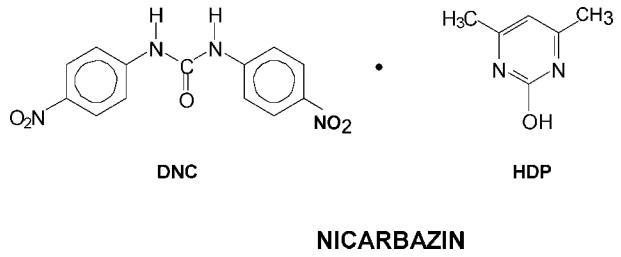

Figure 1. Nicarbazin.

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poultry since the mid-1950s.[1] However, when fed to breeder or layer hens, nicarbazin reduces egg hatchability and/or egg production. Adsorption, distribution, metabolism and excretion studies in chicken indicate that DNC is the bioactive component of nicarbazin. While both DNC and HDP are absorbed, 98% of HDP is excreted within 24 h, predominantly via the urine. DNC has a longer half-life, 94% being excreted in 4 days, predominantly via the feces. Tissue and egg residues of DNC are generally 10 to 50 times greater for DNC than for HDP.[2] These data suggest that DNC residues are more applicable than those of HDP for use as a marker of nicarbazin ingestion.

Multiple research studies have shown that the addition of nicarbazin to diets of females resulted in reduced egg production, hatchability, egg weight, shell pigmentation and increased yolk mottling.[1,3–6] Given its long history of safe use as a coccidiostat in chickens, we feel that nicarbazin is a promising contraceptive agent for pest avian wildlife species such as Canada Geese. However, conducting feeding and hatchability studies with wild species such as Canada Geese is tedious, due to the difficulty of breeding these geese in captivity. To expedite the research process, we have evaluated the effects of multiple dose levels of nicarbazin on the production and hatchability of chicken eggs. Blood and egg concentrations of DNC were also determined. Blood DNC levels were correlated to various levels of contraceptive efficacy. Subsequent studies are being conducted with smaller numbers of ducks and geese to determine the nicarbazin dose required to produce equivalent blood DNC levels in target pest species. This should then permit the expeditious evaluation of multiple formulations and dosing regimes by monitoring blood DNC concentrations in relatively small numbers of the target species, ultimately leading to a promising formulation for field testing. Also, estimation of egg DNC concentrations at the target dosing levels will permit us to determine potential non-target hazards resulting from egg predation. This paper summarizes the results of the chicken nicarbazin feeding study.

2 MATERIALS AND METHODS

2.1 In-life experiment

The 35-day in-life portion of this experiment was conducted at the University of Georgia, Poultry Science Department, Athens, GA. Commercial laying chickens were housed in individual cages in a controlled environment with 16 h of light per day. A total of 100 White Leghorn hens at 55 weeks of age were randomly selected for this experiment. The hens were randomly divided into five groups of 20 birds each. As summarized in Fig 2, the in-life portion of the experiment consisted of a 7-day pre-treatment period, a 14-day treatment period, and a 14-day post-treatment period. Egg production and egg weight were recorded daily for all hens. Yolk mottling was also assessed daily by candling. Hens were artificially inseminated on day 1 and day 3 of the pre-treatment period, and at weekly intervals thereafter.

PRE-TREATMENT	TREATMENT		POST-TREATMENT
1 WEEK	2 WEEKS		2 WEEKS
CONTROL FEED	NICARBAZIN FORTIFIED FEED 0,25, 50,100,150 mg kg^{-1}		CONTROL FEED
PRE-HATCH	HATCH 1	HATCH 2	HATCH 3

Figure 2. In-life study design.

During the 7-day pre-treatment period, all hens were fed a commercial layer ration. Eggs laid on days 4–7 were used for obtaining pre-treatment hatchability data. Eggs and blood samples were collected on day 7 of the pre-treatment period and assayed for DNC to determine baseline residue concentrations and/or the presence of chromatographic interferrants. Blood hemolysis was minimized by collecting blood in capillaries containing 1% EDTA. Plasma was prepared by centrifugation. Plasma and eggs were stored at −15 °C until analyzed.

During the 14-day treatment period, hens were fed diets containing nominal concentrations of 0, 25, 50, 100 or 150 mg kg^{-1} nicarbazin. Blood samples and all eggs were collected at 2-day intervals. Two blood samples were collected from each treatment group on each day of 6 sampling days.[2–6] Five blood samples were collected on each day of sampling days 8–14. Two additional eggs were collected from each treatment group on day 13 of the treatment period and day 1 of the post-treatment period. The DNC content was determined in all the blood and two eggs from each sampling day. Percentage hatchability was assessed in the remaining eggs. During the 14-day post-treatment period, the hens were fed nicarbazin-free commercial layer ration. Blood and egg collection continued as during the treatment period.

2.2 Hatchability and reproduction rate

Eggs were set four times during the experiment. The first and second sets consisted of eggs collected during the pre-treatment period and during the first 6 days of the treatment period, respectively. The third set consisted of eggs produced from day 7 of the treatment period to day 3 of the post-treatment period. The fourth set consisted of eggs collected from days 4–14 of the post-treatment period. Incubation of eggs was initiated 2–3 days after the last collection day. Hatchability was determined for each treatment group and incubation period as the fraction of set (or incubated) eggs that hatched. Reproduction rate was calculated by multiplying the total number of eggs laid per day by the hatchability.

Nicarbazin diet concentration (mg kg^{-1})		Egg residues (DNC, mg kg^{-1})		Plasma residues (DNC, mg kg^{-1})	
Nominal	Actual	Least square means	Rank[a]	Least square means	Rank[a]
0	0	0.00	C	−0.02	D
25	34.9	1.94	C	0.87	C
50	54.2	4.26	B	2.11	B
100	92.5	5.98	A, B	3.18	A
150	147	7.06	A	4.29	A

Table 1. Multiple comparison of DNC egg and plasma residues

[a] Least square means with same letter rank are not significantly different ($P > 0.05$).

2.3 Feed preparation and analysis

Nicarbazin-fortified feeds were prepared by the University of Georgia Poultry Science Department (Athens, GA) at nominal concentrations of 0, 25, 50, 100 and 150 mg kg^{-1}. The nicarbazin content of the feed was subsequently quantified by ultraviolet absorbance (430 nm) following extraction with *N*,*N*-dimethylformamide and an alumina column clean-up (AOAC method 956.11).

2.4 Blood and egg analyses

High-performance liquid chromatography (HPLC) was used to quantify DNC in blood plasma and eggs.[7,8] For plasma analysis, plasma (100 µl) was combined with acetonitrile (200 µl), vortex mixed and centrifuged at 16 000 *g* for 5 min to precipitate proteins. For egg analysis, aliquots of homogenized egg (5 g) were vortex mixed with acetonitrile + *N*,*N*-dimethylformamide (1 + 1 by volume; 7 ml). The mixture was sonicated and centrifuged. The supernatant was then removed and filtered (45 µm). This procedure was repeated twice more with the addition of acetonitrile + dimethylformamide (7 ml) each time. Solvents were combined in a 25-ml volumetric flask and brought to volume with acetonitrile + dimethylformamide. DNC in the plasma and egg supernatants was quantified by reversed-phase HPLC with UV detection (347 nm).

2.5 Statistical analysis

A one-factor ANOVA was conducted with diet = factor, day = covariate and DNC residue concentration = response. Multiple comparisons of least-square means were conducted using the *p*-diff option in SAS.[9] For each treatment group, reproduction rate (compared with control) and egg and plasma DNC residues were plotted *versus* day. DNC residues in eggs *versus* plasma were correlated via linear regression analysis.[10]

3 RESULTS AND DISCUSSION

The actual concentrations of nicarbazin in the treated diets varied from the nominal concentrations by 2 to 40% (Table 1). However, the actual nicarbazin concentrations afforded the desired range of concentrations to evaluate the avian contraceptive efficacy of nicarbazin-fortified feed.

The mean residue data plotted in Fig 3 suggest that egg and plasma DNC residues increased with increasing nicarbazin diet concentrations. As expected, no DNC residues were detected in samples collected from the control group. Also, DNC residues in samples collected from chickens fed 92.5 or 147 g kg^{-1} nicarbazin fortified feed were very similar. These observations were confirmed by the results of the one-factor ANOVA which indicated that diet effects were highly significant with respect to both egg ($F_{4,154} = 17.02$; $P < 0.0001$) and plasma ($F_{4,192} = 40.17$; $P < 0.0001$) DNC residues. Multiple comparisons of least-square means were conducted using the *p*-diff option in SAS[9] and are summarized in Table 1. These analyses substantiated the trend of increasing egg and plasma DNC concentrations with increasing nicarbazin diet concentrations. DNC residues in eggs collected from chickens fed 92.5 and 147 mg kg^{-1} nicarbazin diets were significantly greater than the residues in eggs collected from chickens fed diets containing 34.9 or 0 mg kg^{-1} nicarbazin. The mean

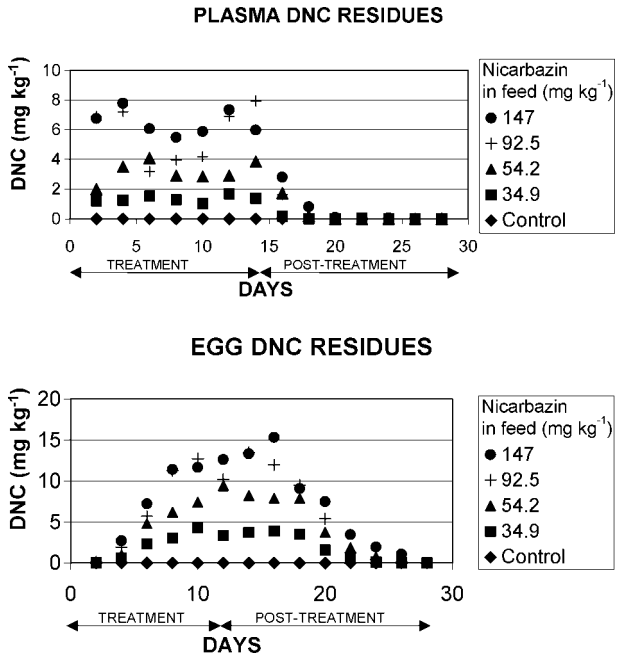

Figure 3. Mean plasma and egg DNC residues.

egg DNC residues for the 147 mg kg^{-1} treatment group were greater than the 54.2 mg kg^{-1} treatment group. Moreover, the mean egg residues in the 147 *versus* 92.5 mg kg^{-1} treatment groups and the 92.5 *versus* 54.2 mg kg^{-1} treatment groups were not significantly different. When the plasma DNC concentrations were examined, the mean residues in the 34.9, 54.2, 92.5 and 147 mg kg^{-1} treatment groups were all significantly greater than the control (0 mg kg^{-1}). Mean DNC plasma concentrations were significantly different for each treatment group, except for the 92.5 *versus* 147 mg kg^{-1} treatment group.

A decrease in production (number of eggs laid) and/or hatchability (eggs hatched/eggs laid) would contribute to decreased reproductive rate. Conversely, an increase in egg production could negate the effects of decreased hatchability on the overall rate of reproduction. For this study, we calculated reproduction rate (relative to control) as an indicator of contraceptive efficacy. The data summarized in Fig 4 indicate that decreased reproduction rate associated with nicarbazin treatments was the result of changes in hatchability or changes in egg production and hatchability. For example, in the chickens fed 54.2 or 92.5 mg kg^{-1} fortified feed, the reproduction rate decrease was solely attributed to a decrease in hatchability. The decreased reproduction rate noted in the chickens fed 34.9 or 147 mg kg^{-1} nicarbazin-fortified feed resulted from the combined effects of decreased hatchability and decreased reproduction rate.

Figure 5 summarizes reproduction rate and mean egg and plasma DNC concentrations with respect to each sampling day of the study for the 0, 54.2 and 147 mg kg^{-1} treatment groups. Data for the 92.5 mg kg^{-1} treatment group (not shown) were similar to those for the 54.2 mg kg^{-1} treatment group. No DNC residues were observed in plasma or eggs collected from the control (0 mg kg^{-1}) group. For the nicarbazin-treated groups, the greatest mean plasma DNC concentrations were observed at days 12 or 14, the end of the treatment period. Maximum mean plasma concentrations of 4.1 and 7.8 mg kg^{-1} were observed for the 54.2 and 147 mg kg^{-1} treatment groups, respectively. Plasma DNC concentrations rapidly decreased upon withdrawal of the nicarbazin-treated feed. Mean plasma concentrations for all groups reached undetectable levels by day 5 of the post-treatment period.

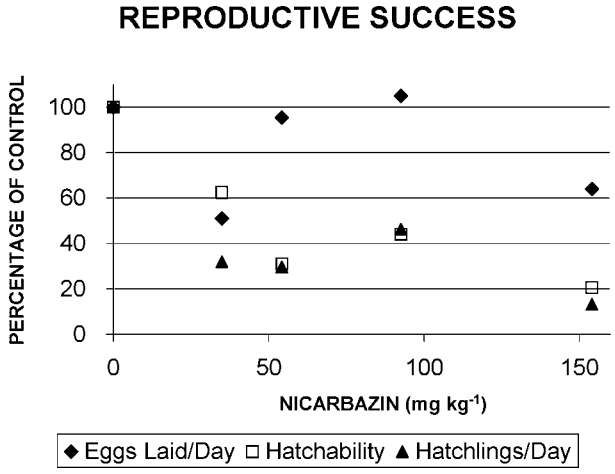

Figure 4. Nominal nicarbazin diet concentrations *versus* relative (to control) egg production, hatchability and reproduction rate during hatch 2 (treatment day 7 to post-treatment day 5).

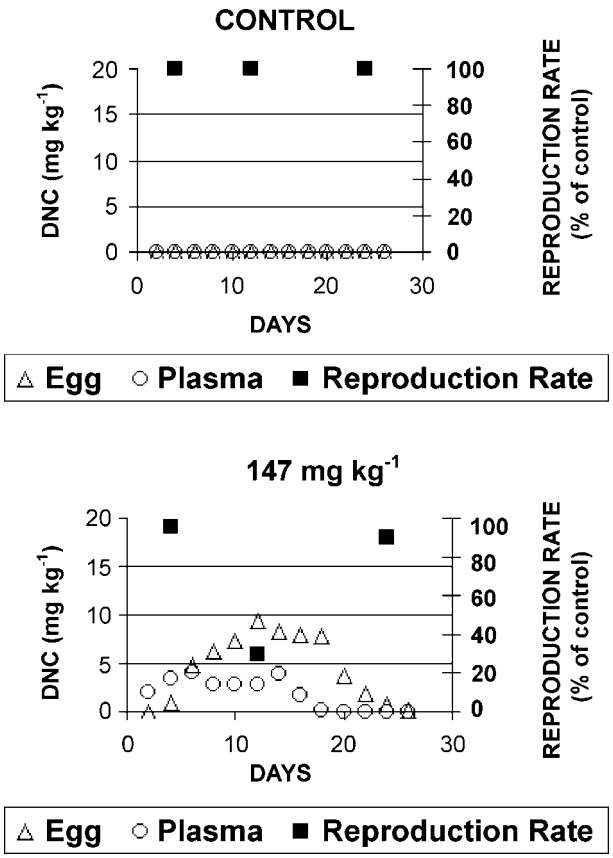

Figure 5. Reproductive rate (relative to control) *versus* mean egg and mean plasma DNC residues.

The trends for plasma and egg residues were similar. For the nicarbazin-treated groups, maximum egg residues were observed several days after plasma residues had maximized. For the first 4 days of the treatment period, plasma residues were greater than egg residues. At day 6 of the treatment period, plasma and egg residues were nearly identical for all groups. Thereafter, mean egg residues exceeded plasma residues for the duration of the treatment period. As the nicarbazin diet concentration increased, egg DNC residues peaked at later times. Maximum egg residues for the 34.9 mg kg^{-1} group were observed on treatment day 10. Egg residues peaked on treatment day 12 for the 54.2 mg kg^{-1} group, on day 2 of the post-treatment period for the 92.5 mg kg^{-1} group and on day 4 of the post-treatment period for the 147 mg kg^{-1} group. Maximum mean egg DNC concentrations were 4.3, 9.4, 13.9 and 15.3 mg kg^{-1} for the chickens consuming feed treated with nicarbazin at 34.9, 54.2, 92.5 and 147 mg kg^{-1}, respectively. Egg DNC resi-

dues decreased more slowly than did plasma DNC residues. For the 34.9 mg kg^{-1} group, egg DNC concentrations did not reach undetectable concentrations until day 10 of the post-treatment period. For the 54.2 and 92.5 mg kg^{-1} groups, DNC egg residues were detected until day 12 post-treatment. For the 147 mg kg^{-1} group, low DNC residues (0.6 mg kg^{-1}) were detected in eggs harvested 14 days post-treatment.

During days 1–6 of dosing, reproduction was suppressed by approximately 33% in chickens consuming 147 mg kg^{-1} treated feed. Reduction of reproduction was minimal in the other treatment groups during this time period. For all groups consuming nicarbazin-treated feed, the lowest reproduction rates were observed during the second time period (day 7 of treatment to day 5 post-treatment). For this time period, the reproduction rate of chickens fed 34.9, 54.2 or 92.5 mg kg^{-1} nicarbazin-treated diets was suppressed by approximately 67%. The reproductive rate of chickens fed 147 mg kg^{-1} nicarbazin fortified feed was suppressed by about 85%.

For the chickens consuming 34.9, 54.2 or 92.5 mg kg^{-1} nicarbazin-fortified feed, plasma DNC concentrations plateaued at approximately 2–4 mg kg^{-1} during treatment days 6–14. For the chickens consuming the 147 mg kg^{-1} nicarbazin fortified diet, plasma DNC concentrations plateaued at 6–8 mg kg^{-1} during treatment days 6–14. These plasma DNC concentrations represent the target concentrations for evaluating nicarbazin diets in pest avian species such as Canada Geese. While absorption, distribution, metabolism and excretion rates may vary between species, it is likely that nicarbazin treatment resulting in plasma levels ranging from 2 to 6 mg kg^{-1} for a duration of approximately 1 week will result in a suppression of reproduction by 66–85%.

In a subsequent study, chickens and geese were fed a 125 mg kg^{-1} nicarbazin-fortified diet for 1 week. The maximum DNC blood levels in geese were approximately half of the level observed for chickens.[7] This suggests that, to achieve plasma DNC levels of 6–8 mg kg^{-1} in geese and hopefully a concurrent 85% decrease in reproduction rate, a diet fortification level of approximately 300 mg kg^{-1} nicarbazin is required.

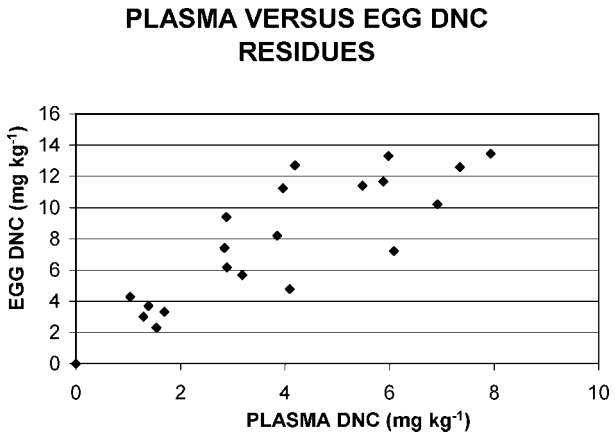

Figure 6. Mean plasma DNC residues *versus* mean egg DNC residues for treatment day 6 to post-treatment day 2.

Figure 3 indicates that the plasma and egg residues for each treatment group generally plateaued between treatment day 6 and post-treatment day 2. In Fig 6, the mean egg residues for each treatment group are plotted *versus* the corresponding mean plasma residue for this time period. Figure 6 illustrates that the relationship between egg and plasma residues for treatment days 6–14 is linear. The value of R^2 for this relationship is 0.83. The linear regression equation

$$(\text{mg kg}^{-1}\text{eggs}) = 1.71 \times (\text{mg kg}^{-1}\text{plasma}) + 0.94$$

provides a means to predict egg DNC residue levels after 1 week of nicarbazin administration. This relationship suggests that plasma DNC analysis can be used to provide an estimate of the maximum anticipated DNC residues in eggs. For example, the maximum plasma residue for the 147 mg kg^{-1} treatment group was 8.4 mg kg^{-1}. According to the linear regression equation, this would equate to a maximum egg residue of 15.7 mg kg^{-1}. The observed maximum egg residue for this treatment group was 15.3 mg kg^{-1}. For the chickens consuming 34.9 mg kg^{-1} feed, the maximum egg residue predicted by the linear regression equation was 4.0 mg kg^{-1}. The observed maximum egg DNC concentration was 3.9 mg kg^{-1}.

4 CONCLUSION

The correlation of blood and egg DNC residues to the contraceptive efficacy of nicarbazin treatments provides an approach to facilitate development of a nicarbazin-based contraceptive for pest waterfowl. By monitoring blood DNC levels in pest species, formulations can be evaluated efficiently in approximately 2 weeks. In addition, such studies can be conducted throughout the year. This offers a tremendous increase in research efficiency over evaluating the contraceptive efficacy of formulations under field conditions. Such field studies require large numbers of birds, several months and may only be conducted once a year (during breeding season). Additionally, the quantification of blood and egg DNC residues may also be used to facilitate the ultimate field testing of a promising nicarbazin formulation. Blood samples may be obtained and analyzed to determine the percentage of the pest waterfowl that are in fact consuming the bait and/or how much bait the subjects are consuming. These blood concentrations can also be used to predict the DNC residues in the eggs that will produced during the field study. These egg residues may be important for predicting potential secondary hazards associated with conducting field evaluations of nicarbazin. Moreover, in field studies where obtaining blood from study subjects is undesirable or not possible, egg residues may be used determine the associated plasma residues. These predicted plasma

levels can be subsequently correlated to bait consumption.

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